Estradiol Metabolites Attenuate Renal and Cardiovascular Injury Induced by Chronic Nitric Oxide Synthase Inhibition

Stevan P. Tofovic,† Eman M. Salah,‡ Raghvendra K. Dubey,*† Mona F. Melhem,‡ and Edwin K. Jackson*§*

Abstract: Our previous studies in rodent models of nephropathy demonstrate that 2-hydroxyestradiol (2HE), an estradiol metabolite with little estrogenic activity, exerts renoprotective effects. In vivo, 2HE is readily converted to 2-methoxyestradiol (2ME), a major estradiol metabolite with no estrogenic activity. The goal of this study was to determine whether 2ME has renal and cardiovascular protective effects in vivo. First, the acute (90 minutes) and chronic (14 days) effects of 2ME (10 μg/kg/h) on blood pressure and renal function were examined in normotensive and spontaneously hypertensive rats (SHR). Second, a rat model of cardiovascular and renal injury induced by chronic nitric oxide synthase inhibition (N^{ω}-nitro-L-arginine; 40 mg/kg/d; LNNA group) was used to examine the protective effects of estradiol metabolites. Subsets of LNNA-treated rats were administered either 2HE or 2ME (10 μg/kg/h via osmotic minipump; LNNA+2ME and LNNA+2HE groups, respectively. 2-Methoxyestradiol had no acute or chronic effects on blood pressure or renal function in normotensive animals or on hypertension in SHR. Prolonged, 5-week NOS inhibition induced severe cardiovascular and renal disease and high mortality (75%, LNNA group). 2ME, but not 2HE, significantly decreased elevated blood pressure and attenuated the reduction in GFR. 2HE delayed the onset of proteinuria, whereas no proteinuria was detected in the 2-ME group. 2HE and 2ME reduced mortality rate by 66% and 83%, respectively ($P < 0.001$). In the kidney, 2HE and 2ME abolished LNNA-induced interstitial and glomerular inflammation, attenuated glomerular collagen IV synthesis, and inhibited glomerular and tubular cell proliferation. In the heart, 2HE and 2ME markedly reduced vascular and interstitial inflammation and reduced collagen synthesis and vascular/interstitial cell proliferation. This study provides the first evidence that, in a model of severe cardiovascular and renal injury, 2-methoxyestradiol (a major nonestrogenic estradiol metabolite) exerts renal and cardiovascular protective effects and reduces mortality.

Key Words: estradiol, metabolites, cardiovascular disease, chronic renal failure

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Accelerated arteriosclerosis and excessive rate of cardiovascular morbidity and mortality characterize chronic renal disease (CRD).[1] The burden of cardiovascular disease (CVD) in the CRD population includes left ventricular hypertrophy and dilation, myocardial fibrosis, ischemic heart disease, peripheral vascular disease, and hypertension.[2,3] Importantly, a growing body of evidence indicates that processes contributing to CVD commence early in the progression of CRD and suggests that early intervention may reduce the burden of cardiovascular disease in the CRD population. Similar to cardiovascular disease, the incidence and prevalence of CRD are higher in men than in women.[4] Although the involvement of genetic factors, environment, and androgens should not be neglected,[5,6] the resistance of kidneys in women to the progression of renal disease is most frequently attributed to estrogens.[7]

Previous experimental and clinical data suggested that estrogens may provide renal and cardiovascular protection and that, therefore, hormone replacement therapy would be a rational approach to retard the loss of renal function and attenuate cardiovascular morbidity/mortality in populations with chronic renal disease. However, recent large prospective clinical studies (HERS, Women Health Initiative)[8–10] questioned the beneficial effects of estrogens and indicated that the use of estrogens in fighting cardiovascular disease may not be as effective as thought previously, ie, that the risk of cancer and cardiovascular complications outweighs the potential benefits of hormone replacement therapy. Obviously, the use of estradiol analogues with no estrogenic activity that exhibit cardiovascular and renal protection would provide powerful new weapons for fighting high cardiovascular morbidity and mortality in CRD population. Our recent data and the present study suggest that this may be the case with metabolites of 17β-estradiol.

Previously, we have demonstrated that 2-hydroxyestradiol (2HE) has cardiovascular and renal protective effects in ZSF_1 rats, a genetic model of obesity and nephropathy

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associated with the metabolic syndrome (ie, hypertension, insulin resistance, and hyperlipidemia).[11,12] In obese male ZSF_1 rats, chronic treatment with 2HE improved metabolic status (reduced obesity, severity of diabetes, and plasma cholesterol) and endothelial function, decreased blood pressure and proteinuria, and reduced the extent of tubulointerstitial damage and glomerulosclerosis.[13,14] Furthermore, in a recent study in chronic puromycin-aminonucleoside (PAN)-induced nephropathy model, we demonstrated that treatment with 2HE reduces proteinuria and retards the progression of renal disease, ie, reduces glomerular and interstitial inflammation, mesangial cell activation, and extracellular matrix expansion.[15]

In the bloodstream (by catechol-O-methyltransferase derived from erythrocytes), 2HE is readily converted to 2-methoxyestradiol (2ME), a metabolite with no estrogenic activity.[16–18] The goal of this study was to determine whether 2ME has cardiovascular and renal protective effects in vivo. We used a rat model of renal and cardiovascular injury induced by chronic nitric oxide synthase inhibition. Because there are no data regarding the cardiovascular and renal effects of 2ME in rats, we first studied the acute and chronic effects of 2ME on blood pressure and renal function in normotensive and spontaneously hypertensive rats. This study provides the first evidence that 2-methoxyestradiol (a major nonestrogenic, nontoxic, and noncarcinogenic endogenous metabolite of 17β-estradiol) exerts significant renal and cardiovascular protection in vivo.

MATERIALS AND METHODS

Animals

A total of 58 male Sprague-Dawley rats and 26 male spontaneously hypertensive rats (SHR) were used in this study. Rats were housed in the University of Pittsburgh Medical Center animal care facility (temperature 22°C; light cycle 12 hours; relative humidity 55%). Animals were fed Pro Laboratory RMH 3000 rodent diet (PMI Nutrition Inc, St Louis, MO) and were given water ad libitum. Institutional guidelines for animal welfare were followed, and the Institutional Animal Care and Use Committee approved experimental protocols.

Protocol 1: Effects of 2-Methoxyestradiol on Blood Pressure and Renal Function in Normotensive and Hypertensive Animals

Normotensive Sprague-Dawley rats (n = 6 per group) were anesthetized and instrumented for measurements of blood pressure function as described previously.[15] A midline abdominal incision was made, the left kidney exposed, and a PE-10 catheter was inserted into the left ureter to facilitate collection of urine. A flow probe (Model 1RB; Transonic Systems, Inc, Ithaca, NY) was placed on the left renal artery for measurements of renal blood flow (RBF), and 5-minute averages for mean arterial blood pressure and RBF were used to calculate renal vascular resistance (RVR). Next, an infusion of [^{14}C]inulin (0.035 μCi/20 μL saline/min) was initiated. Animals also received 90-minute intravenous infusion of either saline (50 μL/min, control groups) or 2ME 10 μg/kg/h (2ME groups; 2-ME $t_{½} \sim 20$ minutes)[18] before measurements of blood pressure and renal function were conducted. A blood midpoint and urine [^{14}C]inulin was measured, and renal clearance of [^{14}C]inulin was calculated as an estimate of GFR. Another subset of normotensive animals (n = 7 per group) were implanted with osmotic minipumps (model 2ML2, Alzet, Palo Alto, CA) containing vehicle (polyethylene glycol, PEG-400; 5 μL/h; control) or 2-methoxyestradiol (10 μg/kg/h; 2ME group). Fourteen days later, animals were anesthetized with pentobarbital (45 mg/kg IP) and instrumented for measurements of blood pressure and renal hemodynamics and excretory function. After a 45-minute rest period, two 30-minute clearance periods were conducted. The acute and chronic effects of 2ME on blood pressure were also examined in spontaneously hypertensive rats (SHR; n = 6–7 per group), using the identical protocol as for normotensive animals.

Protocol 2: Effects of Estradiol Metabolites in LNNA-Treated Rats

Before initiating the treatment and 10, 15, 25, and 35 days into the treatments, a total of 32 rats were placed in metabolic cages and allowed to acclimatize for 2 days. Twenty-four-hour measurements of urine volume and food and water intakes were conducted; urine samples were analyzed, and 24-hour urinary sodium, potassium, creatinine, and protein excretions were determined. Tail vein blood samples were also taken for measurement of plasma concentrations of sodium, potassium, and creatinine. Plasma and urine samples were analyzed for sodium and potassium using a flame photometer (Model IL-943; Instrumentations Laboratory Inc, Lexington, MA), and creatinine concentrations were measured with a creatinine analyzer (Creatinine Analyzer 2, Beckman Instrument, Fullerton, CA). Total urine proteins were measured by a spectrophotometric assay using bicinchoninic acid reagent (Pierce; Rockford, IL).

After baseline metabolic parameters were measured, animals were randomly assigned to have free access to powder food (control group, n = 8) or to receive food/drug mixture containing N^{ω}-nitro-L-arginine (40 mg/kg/d; Sigma; LNNA group, n = 8). Animals in control and LNNA groups were implanted with osmotic minipumps (model 2ML4, Alzet, Palo Alto, CA) containing polyethylene glycol (PEG-400, 2.5 μL/h, Sigma). A subset of LNNA-treated rats (n = 16) was implanted with osmotic minipumps containing 2-HE and 2-ME (10 μg/kg/h; LNNA+2HE and LNNA+2ME groups, respectively; n = 8). Animals were selected for treatments by random assignment. Osmotic minipumps were replaced after 25 days of treatment. Both estradiol metabolites were obtained from Steraloids, Inc (Newport, RI). After 35 days of treatment, animals were anesthetized with pentobarbital (45 mg/kg IP) and instrumented for measurements of renal hemodynamics and excretory function, as described in Protocol 1.

Renal and Cardiac Immunohistochemical Studies

Kidneys and hearts were fixed in 10% buffered formalin for subsequent light microscopy and immunohistochemistry. The tissue sample was sectioned and was processed into paraffin blocks for light microscopy. Five-micrometer tissue

sections from formalin-fixed, paraffin-embedded renal cortices and cardiac samples were dewaxed and stained with periodic acid Schiff (PAS) stain for histologic assessment. Kidney and heart slices were examined by light microscopy and were scored in a blinded fashion by one of the investigators (E.S.). Histopathological features were assessed semiquantitatively on 10 high-power fields (×400) and included segmental (FSGS) and global (FGGS) glomerulosclerosis, tubular atrophy ($0–3^+$), interstitial inflammation ($0–3^+$), tubular dilation ($0–3^+$), arterial medial hypertrophy and arteriolar sclerosis ($0–3^+$).

For labeling of collagen IV, 5-μm segments of renal cortex and ventricular myocardium were incubated overnight at 4°C with a polyclonal anti–collagen IV antibody (dilution 1/500; Chemicon International Inc, Temecula, CA). A primary monoclonal mouse antibody (1/200 dilution; Dako, Carpenteria, CA) was used to label proliferating cell nuclear antigen (PCNA). A polyclonal ED1 antibody (Serotec, Raleigh, NC) specific for a monocyte/macrophage cytoplasmatic antigen was used to label glomerular, vascular, and interstitial macrophages. Nonspecific staining was assessed by replacing the primary antibody with phosphate-buffered saline (PBS). Sections were washed and further developed according to the directions of the manufacturer (Dako Corp, Carpinteria, CA) using the LSAB2 kit that contained a second antibody linked to avidin and peroxidase-conjugated biotin. Immunochemical staining for collagen IV and PCNA were assessed quantitatively with a SAMBA 4000 image analyzer (Image Products International, Chantilly, VA) using specialized computer software (Immuno-Analysis, version 4.1, Microsoft, Richmond, WA) and a color video camera. Software designed for immunostaining analysis enabled the operator to set density threshold values by averaging several fields on the negative control tissues in which the primary antibody was replaced with PBS. Background subtraction was then performed automatically on every tissue. Ten high-power fields (×400) were assessed for staining density or positively marked cells for ED-1. The results are reported as the labeling index, which represents the percentage of the total examined area that stained positively. Staining intensity of positive areas was also assessed (mean optical density), and a mean quick score was then calculated (mean optical density × labeling index).

Statistical Analyses

Statistical analyses were performed using the Number Cruncher statistical software program (Kaysville, UT). Group comparison for data from metabolic studies (repeated measurements) were performed by 2-factor, hierarchical

TABLE 1. Metabolic Parameters in Control and LNNA-Treated Animals (LNNA) and in LNNA Rats Receiving 2-Hydroxyestradiol (LNNA + 2HE) or 2-Methoxyestradiol (LNNA + 2ME)

Treatment	N	Body Weight (g)	Food Intake (g/kg/d)	Fluid Intake (mL/kg/d)	Urine Volume (mL/kg/d)	Urine Creatinine (mg/kg/d)	Urine Na^+ (mEq/kg/d)	Urine K^+ (mEq/kg/d)
Baseline								
CONT	8	291 ± 7	81 ± 3	154 ± 15	46 ± 7	49 ± 7	4.39 ± 0.8	12.33 ± 2.3
LNNA	8	309 ± 5	76 ± 1	141 ± 8	46 ± 3	38 ± 3	3.78 ± 0.21	10.49 ± 0.8
LNNA+2HE	8	307 ± 3	78 ± 1	146 ± 12	41 ± 6	41 ± 6	3.95 ± 0.41	11.01 ± 1.10
LNNA+2ME	8	298 ± 2	75 ± 3	148 ± 11	48 ± 5	44 ± 3	4.44 ± 0.46	11.63 ± 0.89
10 days								
CONT	8	321 ± 7	71 ± 2	148 ± 6	43 ± 3	39 ± 3	4.76 ± 0.37	12.68 ± 0.96
LNNA	8	341 ± 6	68 ± 3	101 ± 23	44 ± 5	32 ± 6	4.34 ± 0.61	10.88 ± 1.27
LNNA+2HE	8	318 ± 6	61 ± 4	141 ± 9	50 ± 5	41 ± 6	4.28 ± 0.22	11.99 ± 0.61
LNNA+2ME	8	290 ± 3*	71 ± 4	155 ± 8	51 ± 2	37 ± 2	4.57 ± 0.34	10.57 ± 1.67
15 days								
CONT	8	337 ± 7	83 ± 8	156 ± 7	37 ± 4	37 ± 3	4.53 ± 0.33	11.14 ± 0.61
LNNA	8	355 ± 5	64 ± 2	172 ± 13	65 ± 9	33 ± 4	4.48 ± 0.59	11.13 ± 1.24
LNNA+2HE	8	324 ± 5	64 ± 3	171 ± 8	51 ± 6	31 ± 4	4.19 ± 0.41	12.28 ± 1.43
LNNA+2ME	8	289 ± 5*	69 ± 2	170 ± 14	47 ± 9	33 ± 4	3.75 ± 0.53	9.23 ± 1.16
25 days								
CONT	8	363 ± 10	80 ± 3	146 ± 9	39 ± 6	36 ± 3	4.99 ± 0.63	12.29 ± 1.44
LNNA	5	321 ± 8*	32 ± 10*	173 ± 33	96 ± 33*	25 ± 7	4.77 ± 1.7	12.53 ± 2.95
LNNA+2HE	7	337 ± 6*	60 ± 1*	144 ± 5	42 ± 4	31 ± 4	5.00 ± 0.43	14.9 ± 1.39
LNNA+2ME	8	281 ± 10*	68 ± 11	161 ± 12	54 ± 10	32 ± 3	3.87 ± 0.0.61	9.77 ± 2.01
35 days								
CONT	8	382 ± 11	77 ± 11	133 ± 12	32 ± 6	35 ± 5	4.39 ± 0.56	10.26 ± 1.24
LNNA	3	269 ± 2*	32 ± 10*	267 ± 48*	139 ± 32*	16 ± 2†	5.56 ± 3.96	16.23 ± 12.3
LNNA+2HE	7	314 ± 16*	34 ± 2*	135 ± 19	83 ± 5*	37 ± 3	4.19 ± 0.77	9.99 ± 1.16
LNNA+2ME	7	295 ± 3*	70 ± 10	161 ± 16	51 ± 1*	22 ± 6‡	3.66 ± 0.59	8.43 ± 1.53

ANOVA $P < 0.05$; Fisher LSD test $P < 0.05$ *vs cont, †vs cont and LNNA+2HE, ‡vs LNNA+2HE.

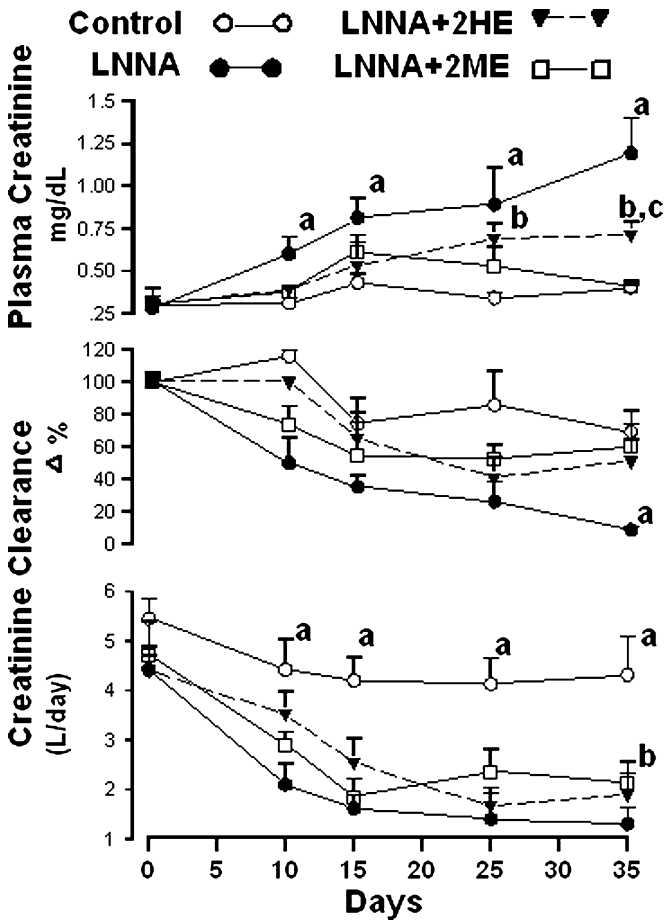

FIGURE 1. Plasma creatinine (upper panel) and creatinine clearance (as percentage change from baseline, middle; as liters per day, lower panel) in control and LNNA-treated animals receiving vehicle (PEG 400, 2.5 μL/min; control, and LNNA groups, respectively) and in LNNA rats treated with 2-hydroxyestradiol (10 μg/kg/h, LNNA+2HE group) or 2-methoxyestradiol (10 μg/kg/h, LNNA+2ME group). Results represent mean ± SEM. Upper panel, 2F-ANOVA: Treatment $P < 0.001$; time $P < 0.001$; treatment–time interaction $P < 0.001$. Middle panel, 2F-ANOVA: Treatment $P < 0.06$; time $P < 0.001$; treatment–time interaction $P = 0.187$. Lower panel, 2F-ANOVA: Treatment $P < 0.001$; time $P < 0.001$; treatment–time interaction $P = 0.180$. Fisher LSD test for post-hoc comparison: (a) $P < 0.05$ versus other groups; (b) $P < 0.05$ versus control group; (c) $P < 0.05$ versus LNNA+2ME group.

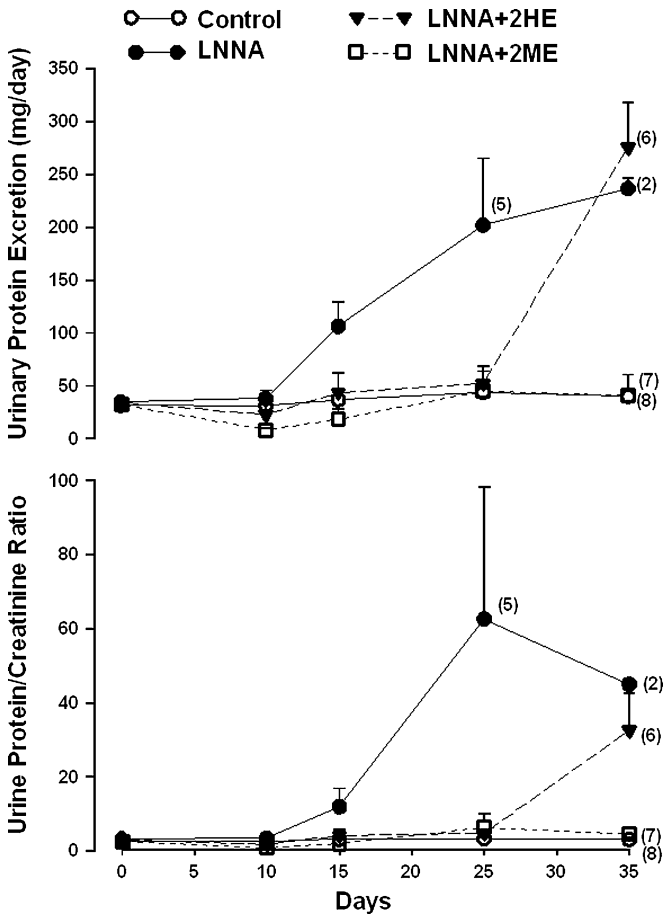

FIGURE 2. Urinary protein excretion and urinary protein/creatinine ratio in control and LNNA-treated animals receiving vehicle (PEG 400, 2.5 μL/min; control and LNNA groups, respectively), and in LNNA rats treated with 2-hydroxyestradiol (10 μg/kg/h, LNNA+2HE group) or 2-methoxyestradiol (10 μg/kg/h, LNNA+2ME group). Results represent mean ± SEM. Upper and lower panels, 2F-ANOVA: Treatment $P < 0.001$; time $P < 0.001$; treatment–time interaction $P < 0.001$.

analysis of variance (2-F ANOVA), followed by Fisher LSD test for post-hoc comparison. 1F-ANOVA was used to compare renal and cardiac histology and renal hemodynamics and excretory function data among all 4 groups. The probability value of $P < 0.05$ was considered statistically significant. All data are presented as mean ± SEM.

RESULTS

In control Sprague-Dawley rats, a 90- to 150-minute infusion of 2ME (10 μg/kg/h) had no effect on blood pressure, renal blood flow, renal vascular resistance, or glomerular filtration rate (MABP 114 ± 2 and 107 ± 3 mm Hg; RBF 8.2 ± 1.4 and 7.3 ± 0.74 mL/min; RVR 16.2 ± 3.0 and 15.3 ± 1.72 mm Hg/mL/min; GFR 3.0 ± 0.2 and 2.9 ± 0.2 mL/min for 2ME and control, respectively). Also, a 2-week administration of 2ME (10 μg/kg/h by osmotic minipump) did not alter blood pressure and renal function (MABP 108 ± 4 and 109 ± 3 mm Hg; RBF 8.4 ± 0.7 and 7.8 ± 0.5 mL/min; RVR 13.7 ± 1.4 and 14.6 ± 1.4; GFR 2.69 ± 0.26 and 3.01 ± 0.3 mL/min; 2ME and control, respectively). 2ME had no acute or chronic effects on mean arterial blood pressure in spontaneously hypertensive rats (90-minute 2ME infusion 166 ± 3 and 164 ± 2 mm Hg; 14-day 2ME treatment 200 ± 4 and 198 ± 6 mm Hg, control and 2ME groups, respectively).

As presented in Table 1, before the beginning of treatments and before induction of cardiovascular and renal injury, experimental groups did not differ with regard to basic metabolic parameters. After 10 days of treatment, in the

TABLE 2. Acute Measurements of Renal Hemodynamics and Excretory Function in Control Animals (Control) and LNNA-Treated Rats Receiving Vehicle (LNNA), 2-Hydroxyestradiol (LNNA+2HE), or 2-Methoxyestradiol (LNNA+2ME) for 35 Days.

Parameters (MSE)	Control (n = 8)	LNNA (n = 3)	LNNA+2HE (n = 6)	LNNA+2ME (n = 7)
BW/Kidney weight (g/mg)	6.37 ± 0.08	8.77 ± 0.1*†	9.05 ± 0.26*†	7.92 ± 0.24*†
Left kidney weight (g)	1.23 ± 0.03	1.43 ± 0.5*†	1.35 ± 0.06*†	1.13 ± 0.04*†
Mean blood pressure	125 ± 4	174 ± 5	181 ± 7	151 ± 6†‡
Renal blood flow (mL/min)	7.4 ± 0.4	1.20 ± 0.05†	0.80 ± 0.15†	3.30 ± 0.50 †‡
Renal plasma flow (mL/min)	3.76 ± 0.31	0.59 ± 0.03†	0.36 ± 0.08†	2.34 ± 0.09†‡
Hematocrit (%)	51 ± 1	51 ± 1	49 ± 1†	46 ± 1†‡
Renal vascular resistance (mm Hg/mL/min)	21.2 ± 1.68	166 ± 7.6†	225.5 ± 7†	66.1 ± 14.1†‡
Urine volume (μL/30 min)	252 ± 39.6	640 ± 43†	215 ± 62†	170 ± 35.5‡
Glomerular filtration rate (mL/min)	4.05 ± 0.23	0.40 ± 0.05†	0.18 ± 0.1†	1.64 ± 0.42†‡

*n = 8.
2F-ANOVA, $P < 0.05$: †vs Control; ‡vs LNNA and LNNA+2HE.

presence of similar food consumption, animals treated with LNNA+2ME but not LNNA or LNNA+2HE had reduced body weight. The effects of 2ME on body weight ($P < 0.005$, 2F-ANOVA) persisted throughout the study. Reduced body weight was also detected in LNNA and LNNA+2HE groups after 25 and 35 days of treatment. However, this reduction was associated with reduced food intake. The latter most likely reflects the severity of cardiorenal injury and poor health of animals in LNNA and LNNA+2HE groups. Similarly, the increases in fluid intake and urine volume after 25 and/or 35 days of treatment in LNNA and LNNA+2HE groups were most likely secondary to concentration defects caused by more severe tubular damage in these animals (see below).

Prolonged and potent NOS inhibition resulted in severe renal and cardiovascular injury. Chronic treatment with high-dose LNNA increased plasma creatinine levels, markedly reduced creatinine clearance, induced proteinuria (Figs. 1 and 2) and severely increased blood pressure (Table 2). Furthermore, acute measurements of renal hemodynamics and excretory function in anesthetized animals (Table 2) revealed markedly reduced renal blood flow (RBF), renal plasma flow (RPF), and glomerular filtration rate (GFR, inulin clearance) and increased renal vascular resistance (RVR) in LNNA-treated rats as compared with control animals with intact kidneys (Table 2). Severe renal and cardiovascular injury resulted in high mortality (75%) in LNNA-treated animals (Fig. 3). Six out of 8 animals died before the end of the study, and therefore, acute measurements of renal and cardiovascular parameters were conducted in only 2 LNNA-treated animals.

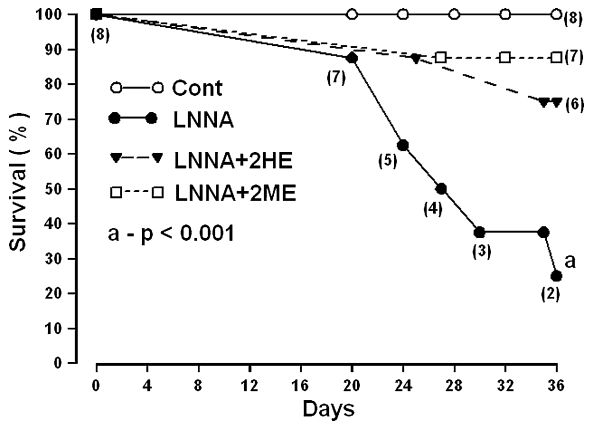

FIGURE 3. Survival curve in control and LNNA-treated animals receiving vehicle (PEG 400, 2.5 μL/min; control and LNNA groups, respectively) and in LNNA rats treated with 2-hydroxyestradiol (10 μg/kg/h, LNNA+2HE group) or 2-methoxyestradiol (10 μg/kg/h, LNNA+2ME group).

Chronic treatment with 2HE attenuated the LNNA-induced increase in plasma creatinine levels, whereas after 35 days of treatment, 2ME and control groups had similar plasma creatinine levels (Fig. 1, top panel). Both metabolites similarly attenuated the decline in creatinine clearance (Fig. 1, middle and bottom panels). Furthermore, 2HE delayed the onset of LNNA-induced proteinuria (Fig. 2, top panel), and this effect become even more significant when urinary protein excretion (UPE) was corrected for creatinine excretion (Fig. 2, bottom panel). 2-Methoxyestradiol had a striking effect on urinary protein excretion. After 10 and 15 days of treatment, 2ME-treated animals had UPE that was even lower ($P < 0.05$) than protein excretion in control animals with intact kidneys, and 2ME prevented LNNA from inducing proteinuria (Fig. 2). Toward the end of the study (25 and 35 days) LNNA- and LNNA+2HE–treated animals had hematuria, whereas, with the exception of 1 LNNA+2ME–treated animal that died on day 28, no hematuria was detected in the LNNA+2ME group. Treatment with 2HE had no effects on the LNNA-induced increase in blood pressure, whereas 2ME significantly reduced the severely elevated blood pressure (Table 2). 2-Hydroxyestradiol had no effects the on LNNA-induced reduction in RBF, RPF, or GFR or increase in RVR. In contrast, 2ME attenuated the LNNA-induced reduction in RBF, RPF, and GFR and the LNNA-induced increase in RVR. It should be mentioned that even though 2ME-treated animals had 3 times higher RVR than control animals, they still had preserved (~40%) filtering capacity (Table 2). Prolonged NOS inhibition with high-dose LNNA resulted in high mortality, and importantly, 2HE and 2ME reduced mortality rate by 66% and 83%, respectively (Fig. 3).

Semiquantitative analysis of kidney samples revealed mild tubular dilatation, presence of proteinaceous material and interstitial and vascular inflammation in LNNA-treated

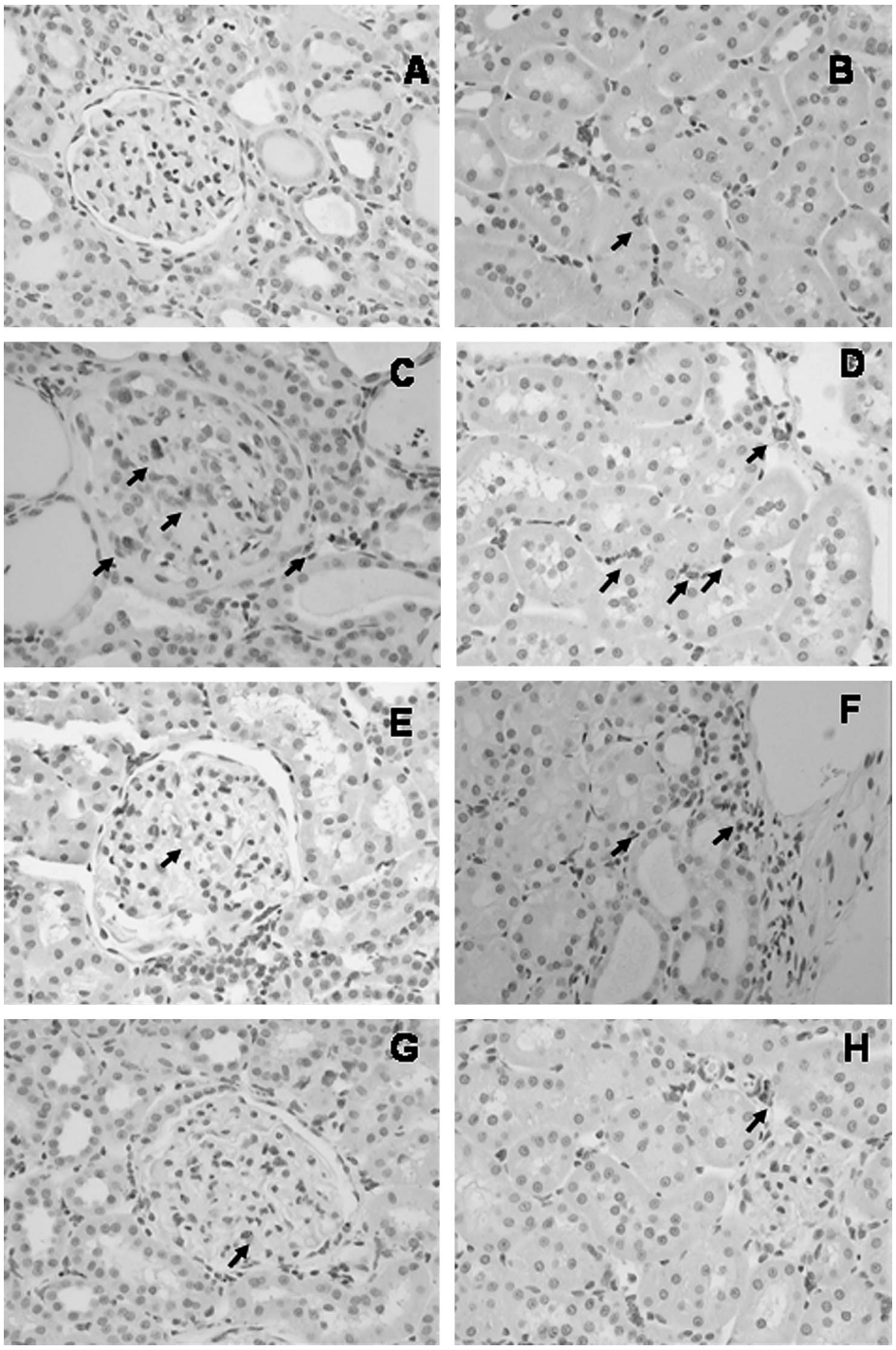

FIGURE 4. Representative immunohistochemistry of cortical sections for ED1$^+$ cells in glomeruli and interstitium of control (A,B) and LNNA nephropathic rat (C,D) and in LNNA-treated animals receiving 2-hydroxyestradiol (E,F) or 2-methoxyestradiol (G,H). Magnification x400.

animals. These changes were reduced in animals treated with estradiol metabolites. Immunohistochemical analysis revealed significant glomerular, interstitial, and tubular changes in LNNA-treated animals. Representative examples of glomerular and interstitial staining for ED1$^+$ cells, PCNA staining in glomeruli and tubules, and collagen IV in glomeruli in renal cortical sections from each study group are shown in Figures 4 to 6. Quantitative assessments of immunohistochemical staining by quantitative image analysis are shown in Figure 7. Prolonged NOS inhibition initiated some of the key mechanisms that, irrespective of the nature of renal injury, lead to glomerular remodeling and sclerosis. Treatment with the NOS inhibitor LNNA induced glomerular and interstitial inflammation (macrophage influx), initiated a proliferative response in both glomeruli and tubules (PCNA), and increased extracellular matrix expansion (collagen IV synthesis).

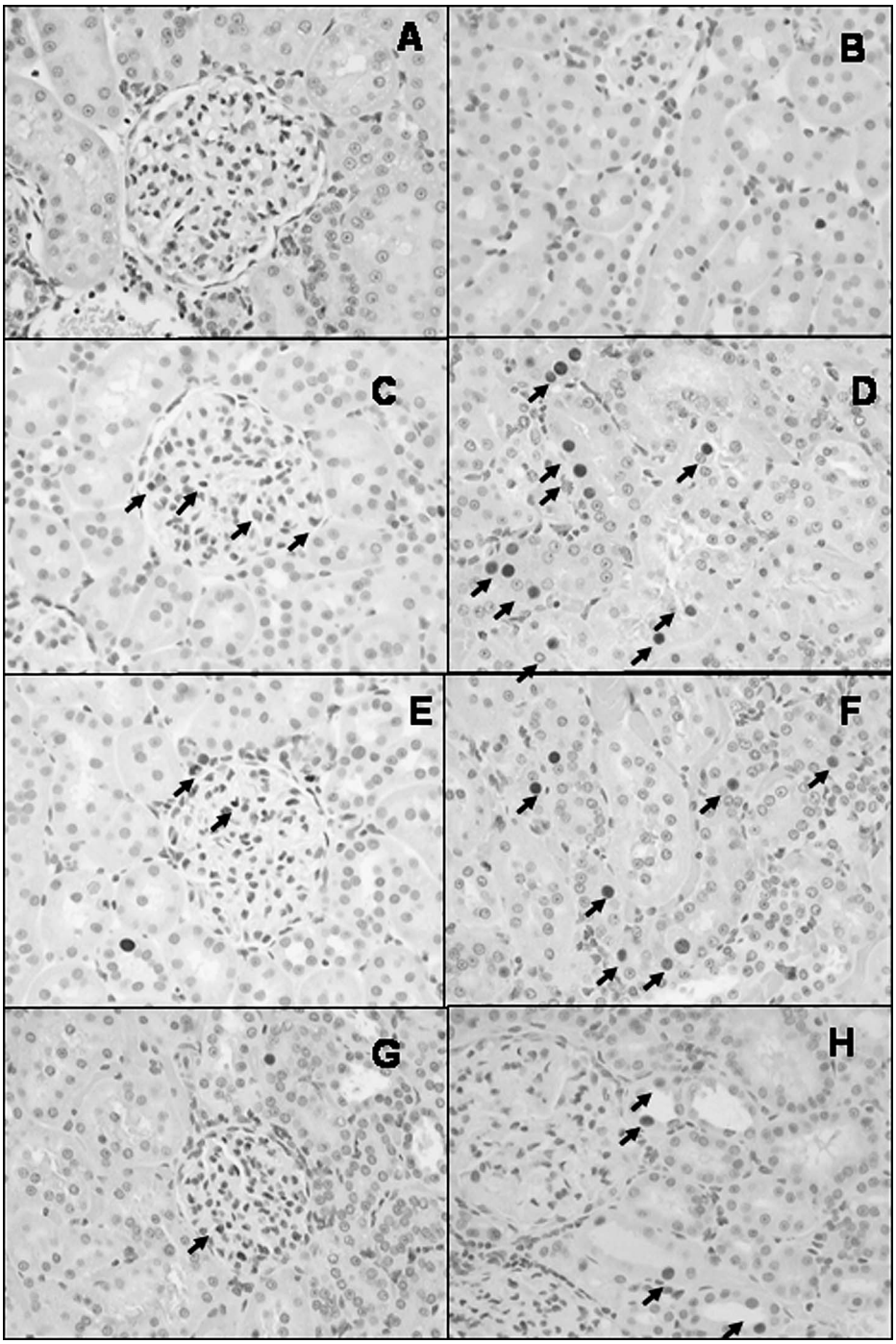

FIGURE 5. Representative PCNA immunohistochemistry of cortical sections in glomeruli and tubules of control (A,B) and LNNA-nephropathic rat (C,D) and in LNNA-treated animals receiving 2-hydroxyestradiol (E,F) or 2-methoxyestradiol (G,H). Magnification x400.

Simultaneous treatment with estradiol metabolites abolished NOS inhibition-induced glomerular and interstitial inflammation (Fig. 7A) and markedly reduced collagen IV synthesis (Fig. 7C). Both metabolites inhibited glomerular and tubular cell proliferation, with 2-ME exhibiting greater antiproliferative effects (Fig. 7B).

Chronic NOS inhibition induced a significant cardiac injury. LNNA-treated rats had cardiac hypertrophy, and the heart/body weight index was 3.10 ± 0.09, 4.45 ± 0.35, 4.20 ± 0.25, and 3.59 ± 0.14 g/kg in control, LNNA, LNNA+2HE, and LNNA+2ME groups, respectively (1F-ANOVA, $P < 0.001$). 2-Methoxyestradiol, but not 2HE, significantly reduced LNNA-induced cardiac hypertrophy. Chronic NOS inhibition also induced cardiovascular inflammation and vascular remodeling. The number of positive ED1 cells and quantitative assessment of immunostaining for PCNA and collagen IV in the hearts from the 4 experimental groups are presented in Figure 8. NOS inhibition produced significant

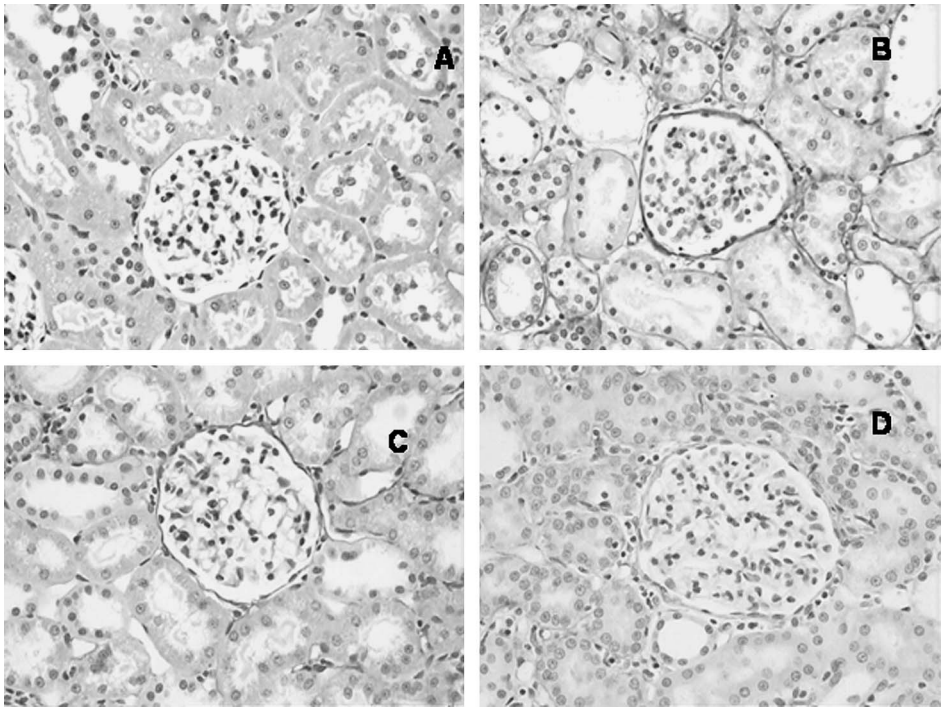

FIGURE 6. Representative immunohistochemistry of cortical sections for collagen IV in glomeruli of control (A) and LNNA nephropathic rat (B) and in LNNA-treated animals receiving 2-hydroxyestradiol (C) or 2-methoxyestradiol (D). Magnification x400.

interstitial and coronary vascular inflammation and increased cell proliferation and collagen IV synthesis. Both 2HE and 2ME markedly reduced vasculitis and interstitial inflammation and reduced collagen synthesis and vascular/interstitial cell proliferation. Importantly, in the heart, 2ME exerted greater antifibrotic and antiproliferative effects.

DISCUSSION

Similarly to previous reports,[19,20] in the present study, administration of high-dose LNNA (40 mg/kg/d) produced severe cardiovascular and renal injury. Furthermore, the prolonged (35-day) NOS inhibition resulted in high mortality (75%).

2-Hydroxyestradiol attenuated both renal and cardiovascular injury and significantly reduced mortality by 66%. This is in accord with the renal and cardiovascular protective effects of 2HE detected in our previous studies in obese diabetic ZSF_1 rats, and in chronic puromycin aminonucleoside nephropathic rats.[13–15] The renal and cardiovascular protective effects of 2ME were even more impressive. In contrast to 2HE, 2ME prevented the development of proteinuria, significantly decreased the elevated blood pressure, and reduced mortality by 83%. It should be mentioned that toward the end of the study, the animals in LNNA and LNNA+2HE groups had reduced food intake (most likely because of the severity of cardiorenal injury and poor health). Because the daily dose of LNNA was not adjusted for reduced food consumption, during the last 10–15 days of the study, the LNNA and LNNA+2HE groups were receiving less LNNA. This renders the detected cardiovascular and renal protective effects of 2ME even more impressive.

The observed effects of estradiol metabolites raise questions regarding the mechanisms of their cardiovascular and renal protection. Based on the known cardiovascular and renal protective effects of estradiol, it is not surprising that estradiol metabolites expressed cardiovascular and renal protection. Several mutually nonexclusive mechanisms have been suggested to contribute to the protective effects of 17β-estradiol (estradiol, E2) against progression of chronic renal disease. This includes its antioxidant effects,[21–23] the inhibitory effects of E2 on the activity of the renin–angiotensin system,[24–27] E2 effects on nitric oxide, prostaglandin, and endothelin-1 synthesis and/or release,[28–30] inhibition of serum-, angiotensin II-, TGF-β-, and endothelin-induced collagen synthesis, and increase in matrix metalloproteinase activity.[31–33] Importantly, in vitro, estradiol metabolites have been shown to inhibit angiotensin II–induced growth and collagen synthesis, to reduce endothelin synthesis, and to produce even greater antioxidant effects than E2 itself.[34–38] Furthermore, recently we have demonstrated that in vivo estradiol metabolites have protective effects against angiotensin II–induced renal and cardiovascular injury[39] and that in the constricted-aorta rat model 2ME inhibits both pressure- and growth factor–induced cardiac and vascular remodeling.[40] Interestingly, it has been reported that in the high-dose LNNA rat model, antioxidants, angiotensin II type-1 receptor antagonists, and endothelin receptor antagonists attenuate hypertension and/or renal and cardiovascular injury.[19,20,41,42] However, it is not clear which mechanisms were involved in the observed protective effects of estradiol metabolites and mechanistic studies of cardiorenal protection of 2ME in vivo are warranted.

2-Methoxyestardiol was more efficacious than 2HE with regard to reducing blood pressure, preventing proteinuria and cardiac hypertrophy, and inhibiting proliferative processes in

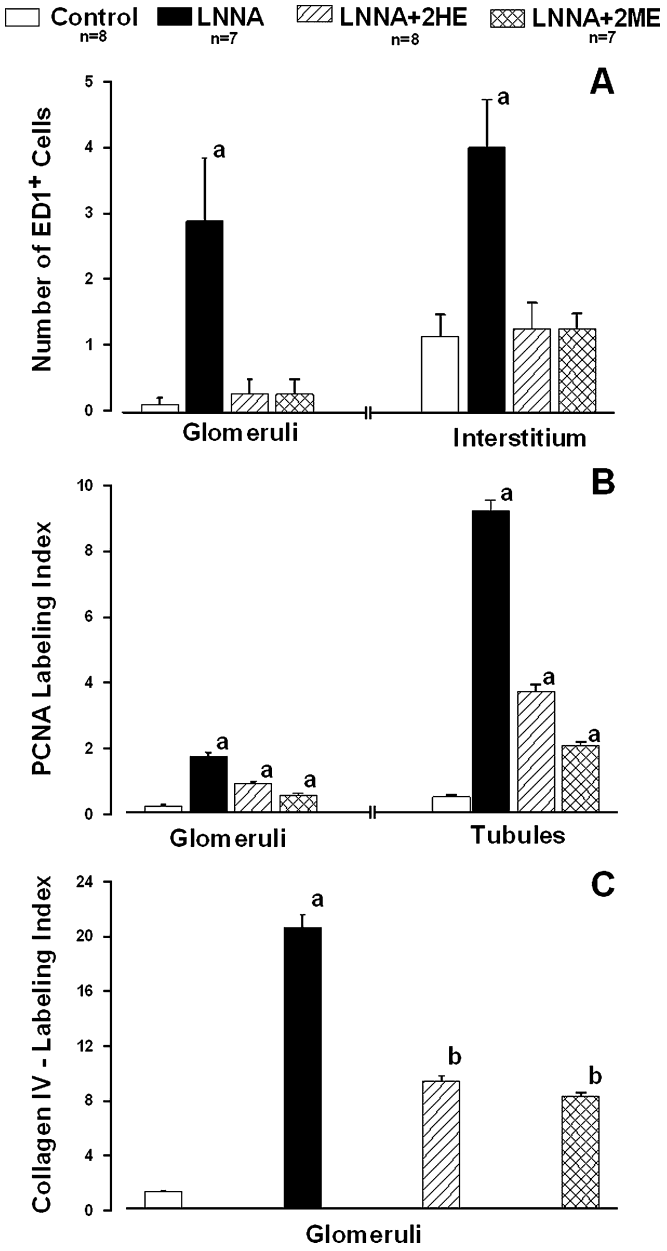

FIGURE 7. Assessment of immunohistochemical staining for ED1+ cells (A), proliferating cell nuclear antigen-PCNA (B), and collagen IV (C) in cortical sections from kidneys of control animals (control), LNNA-nephropathic animals (LNNA), and LNNA-nephropathic animals treated with 2-hydroxyestradiol (LNNA+2HE) or 2-methoxyestradiol (LNNA+2ME). Staining was assessed by image analysis with a SABMA 4000 image analyzer as described in the Materials and Methods section. Results represent mean ± SEM. 1F-ANOVA $P < 0.001$. Fisher LSD test for post-hoc comparison: (a) $P < 0.05$ versus other groups; (b) $P < 0.05$ versus control and LNNA groups.

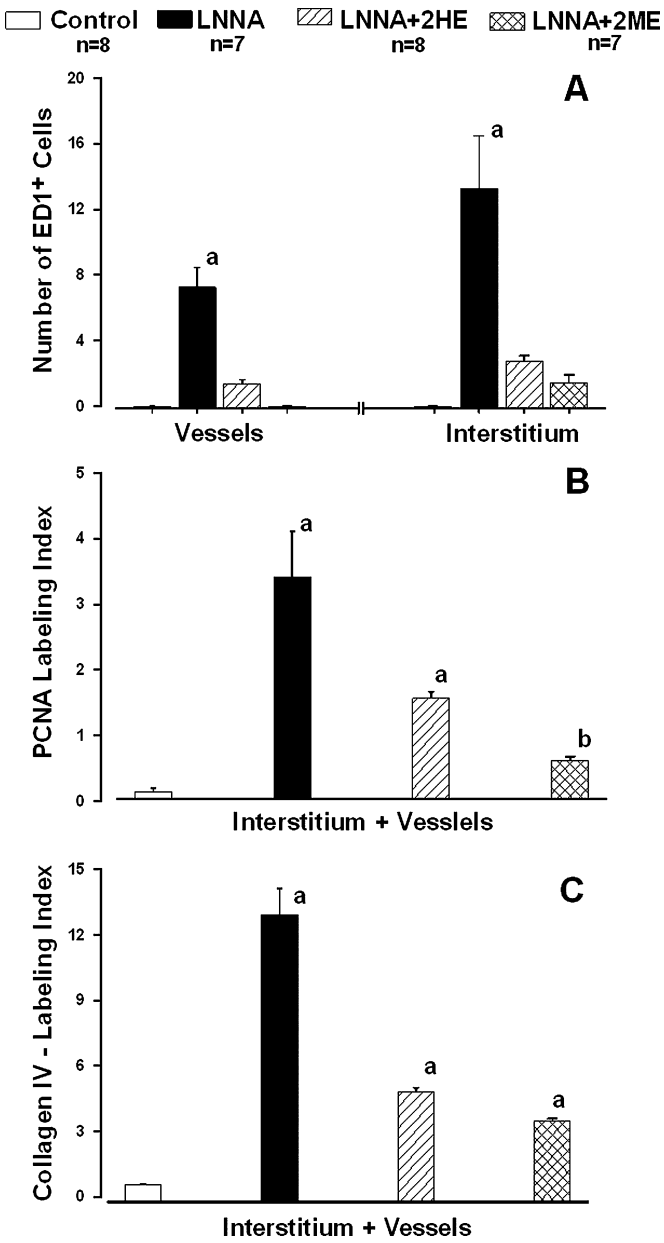

FIGURE 8. Assessment of immunohistochemical staining for ED1+ cells (A), proliferating cell nuclear antigen-PCNA (B), and collagen IV (C) in hearts from control (control) and LNNA-treated animals (LNNA) and LNNA rats treated with 2-hydroxyestradiol (LNNA+2HE) or 2-methoxyestradiol (LNNA+2ME). Staining was assessed by image analysis with a SABMA 4000 image analyzer as described in the Materials and Methods section. Results represent mean ± SEM. 1F-ANOVA $P < 0.001$. Fisher LSD test for post-hoc comparison: (a) $P < 0.05$ versus other groups; (b) $P < 0.05$ versus LNNA and LNNA+2HE groups.

the kidneys and heart. This is somewhat surprising because in vivo 2HE is readily converted to 2ME, and 2HE has a very short half-life of 60–90 seconds.[18,43] The conversion of 2HE largely takes place in the bloodstream, where 2HE is methylated to 2ME by catechol-O-methyltransferase (COMT, enzyme abundantly present in erythrocytes). However, it is possible that in the LNNA model there is reduced conversion of 2HE to 2ME because of elevated catecholamines that competed for COMT. In this regard, it has been reported that in the chronic NOS inhibition model, there is a marked increase

in catecholamines that reaches nanogram levels[44]; in vitro, in mesangial cells, catecholamines inhibit methylation of 2HE and its antimitogenic effects[45]; and in the isolated-perfused kidney catecholamines attenuate 2HE methylation to 2ME.[46] Therefore, the reduced 2HE conversion to 2ME may explain the observed clinical and histopathological differences between the LNNA+2HE and LNNA+2ME groups. However, measurements of both plasma catecholamines and E2 metabolites are required to provide a definitive answer whether plasma catecholamines interfered with 2HE conversion to 2ME. Unfortunately, limited volumes of available blood and high mortality in the LNNA group prevented us from conducting these analyses and from obtaining meaningful information.

In the present study we used daily doses of E2 metabolites (240 μg/kg/d) that were previously shown to bring 2ME plasma concentrations in rats to 2000–4000 pg/mL.[18] These concentrations are 50–100 times higher that those reported in pre- and postmenopausal women.[47] This finding raises questions regarding the physiological relevance and safety of high 2ME concentrations. Nevertheless, 2ME reaches similar, very high concentrations in women during the last trimester of pregnancy.[48] Furthermore, very high daily doses of orally administered 2ME were used safely in animals and in early-phase clinical trials in cancer patients.[49] Importantly, high concentrations of 2ME (similar to those detected in the present study), when used in vitro, exhibit significant (40%–60%) inhibitory effects on vascular smooth muscle cell and cardiac fibroblast growth[46] and on mesangial cell proliferation and collagen synthesis.[16] Furthermore, inhibitory effects of 2ME are not blocked by ICI182780, a highly specific estrogen receptor antagonist, suggesting estrogen receptor–independent mechanisms.[46] Further studies in female rats, using the "low physiological" doses of 2ME, are required to confirm the physiological relevance of the present findings. Nevertheless, present study and available information regarding the physiological/pharmacological properties of 2ME[49] are suggestive of its therapeutic potential.

In summary, this study provides the first evidence that in vivo 2ME, a nontoxic and noncarcinogenic metabolite of estradiol with no estrogenic activity, exhibits significant cardiovascular and renal protection. The protective effects are most likely the result of inhibition of some of the key proliferative mechanisms involved in cardiovascular and glomerular remodeling and sclerosis and are mediated by mechanisms independent of estrogen receptors. Further mechanistic studies of cardiovascular protection of 2-methoxyestradiol in chronic renal failure are warranted.

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